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NADPH-dependent generation of a cytosolic dithiol which activates hepatic iodothyronine 5′-deiodinase

Demonstration by alkylation with iodoacetamide

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We have assessed a previously proposed mechanism mediating 5′-deiodinase activation involving enzymic reduction of disulphides to thiols in non-glutathione cytosolic components of M_r approx. 13000 (Fraction B) catalysed by NADPH in the presence of other cytosolic components of $M_r > 60000$ (Fraction A). The extent of Fraction B reduction under various experimental conditions was monitored by determining the amount of ^{14}C incorporated into chromatographically isolated Fractions B and A after their alkylation with iodo[^{14}C]acetamide. Incorporation of ^{14}C into B was found to require the simultaneous presence of NADPH and A, to be directly proportional to the concentration of NADPH added, and to be unaffected by either propylthiouracil or iopanoate. Activation of 5′-deiodinase attainable using B after its partial reduction by various concentrations of NADPH and subsequent alkylation with non-radioactive iodoacetamide was inversely proportional to the previously added concentration of NADPH. Fraction B was stable at 100 °C for 5 min, while similar heat treatment of Fraction A or omission of NADPH resulted in a complete loss of ^{14}C incorporation. A greater than 90% reduction in iodo[^{14}C]acetamide incorporation was revealed when 0.2 mM-sodium arsenite was added after enzymic reduction of B, as well as when NADPH was replaced by NADH. Fraction B could be labelled more extensively after reduction non-specifically with dithiothreitol or $NaBH_4$, but not by GSH. These observations provide strong evidence for the presence *in vivo* of a cytosolic disulphide (DF_BS_2) in Fraction B which can be reduced enzymically to a dithiol [$DF_B(SH)_2$] by NADPH and cytosolic components in Fraction A. The degree of activation of hepatic 5′-deiodinase correlated with the amount of available (unalkylated) Fraction B.

INTRODUCTION

Microsomes isolated from rat liver homogenates by differential centrifugation have relatively low 5′-iodothyronine deiodinase activity in comparison with that of the whole homogenate, but are stimulated by the addition of cytosol, indicating that cytosol contains an activating cofactor [1–5]. The decline in this supportive activity observed in liver cytosols obtained from rats deprived of food for several days confirmed the original observation of cofactor activity and suggested that *in vivo* this activity was influenced by diet [2,4]. The finding that thiols, particularly DTT, can replace the cytosolic cofactor [1,5], led to the proposal that the cofactor was GSH, and that the intracellular level of GSH was regulated by the availability of NADPH [2,6]. However, the physiological relevance of GSH could not be supported by dietary manipulation of hepatic GSH levels [7] or experiments in which the GSH concentration in cultured hepatocytes was drastically reduced without significant reduction of deiodinase activity [8]. Other experiments in which NADPH or GSH were added to stored homogenates indicated that NADPH had a stimulatory effect on microsomal deiodinase quite apart from its involvement in the reduction of GSSG by glutathione reductase [9]. Hence, as reviewed in detail elsewhere [10,11], the precise role of GSH in relation to the regulation of 5′-deiodination *in vivo* has been controversial.

A recent report [12] from our laboratory presented evidence for the presence among rat liver cytosolic components of a cofactor (designated DF_B) with M_r approx. 13000 (Fraction B) which, acting in conjunction with NADPH and a second cytosolic component, designated DF_A, (with $M_r > 60000$), greatly stimulated 5′-deiodination of iodothyronine by washed hepatic rat microsomes. In additional studies, it was also shown that the apparent maximal stimulatory effects of DTT on 5′-deiodination of rT_3 can be greatly enhanced by the addition of cytosolic fractions containing DF_B [13]. A similar effect was observed by replacing Fraction A and NADPH (required for enzymic reduction) with another dithiol, dihydrolipoamide and, to a lesser extent, with monothiols such as 2-mercaptoethanol and GSH [13]. These studies [12–14] provided indirect evidence that DF_B could act as an efficient intermediate in enhancing 5′-DI activation through the conversion of oxidized hepatic microsomal deiodinase (ESI) to its active

Abbreviations used: DTT, dithiothreitol; 5′-DI, 5′-deiodinase; rT_3, reverse tri-iodothyronine; DF_A, 5′-DI cytosolic reductase in Fraction A; DF_B, 5′-DI cytosolic cofactor in Fraction B; PB-EDTA, 0.125 M-potassium phosphate/1 mM-EDTA, pH 7.4; PTU, 6-*n*-propyl-2-thiouracil; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-[2-(5-phenoxazolyl)]benzene; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

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reduced state (ESH) and supported the theory that cytosolic Fraction B contained a disulphide (DF_BS_2) which could be enzymically reduced *in vivo* in the presence of a reductase (DF_A) in Fraction A and NADPH to produce a dithiol [$DF_B(SH)_2$] as the active form of the cofactor [13] (see Eqn. 1):

$$DF_BS_2 + NADPH + H^+ \xrightarrow{DF_A} DF_B(SH)_2 + NADP^+ \quad (1)$$

The present report concerns studies undertaken to investigate further this postulate by monitoring the extent of Fraction B reduction under various experimental conditions by alkylation with iodo[^{14}C]acetamide (Eqn. 2) to determine thiol production in terms of ^{14}C incorporation into cytosolic Fractions A and B following their separation by chromatography on Sephadex G-50:

$$DF_B(SH)_2 + 2\ I\text{-}{}^{14}CH_2CONH_2 \rightarrow DF_B(S\text{-}{}^{14}CH_2CONH_2)_2 + 2\ HI \quad (2)$$

The experimental data to be shown indicates that the reduced (active) form of DF_B is a dithiol which is enzymically generated in proportion to the amount of NADPH added and that the degree of activation of hepatic 5′-DI is correlated with the amount of residual (unalkylated) DF_B present in Fraction B.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats aged 7–11 weeks and maintained on Purina Laboratory Chow (Ralston Purina, St. Louis, MO, U.S.A.) were used. Iodo[^{14}C]-acetamide (0.1 mCi/ml, 760 μg/ml, radioactivity 24.4 mCi/mmol) was obtained from New England Nuclear and outer-ring-labelled (3′ or 5′) reverse tri-[^{125}I]iodothyronine ([^{125}I]rT_3, 100 μCi/ml, sp. radioactivity > 1200 μCi/μg), Triton X-100 and the scintillants PPO and POPOP were from Amersham International. Sephadex G-50 (fine) was purchased from Pharmacia, and NADPH, NADH, DTT, Nbs_2, unlabelled iodoacetamide and PTU were from Sigma Chemical Co. $NaBH_4$ and $NaAsO_2$ were from Fisher Scientific. Iopanoic acid, a kind gift from Sterling–Winthrop Research Institute (a division of Sterling Drug Inc., Rensselaer, NY, U.S.A.) was used as a sodium salt by dissolving it in dilute NaOH.

Preparation of cytosol and separation of Fractions A and B by fractionation on Sephadex G-50

Cytosol from rat liver was prepared by standard differential centrifugation at 4 °C as described previously [12] and if not immediately used, was stored at −60 °C for not more than 4 weeks. Microsomal pellets obtained by the above procedure were washed three times with PB-EDTA followed by homogenization and centrifugation, resuspended in the same buffer (1 ml/g of starting tissue) and stored at −60 °C.

Column chromatographic fractionation of cytosol on Sephadex G-50 and 5′-iodothyronine deiodinase assay of each fraction were performed essentially as described earlier [12]. To summarize, 80 ml of cytosol (containing approximately 1.6 g of protein) was applied to a 5.0 cm × 80 cm Sephadex G-50 column and eluted at 2.0 ml/min with PB-EDTA. The absorbance of the fractions was monitored at 280 and 415 nm. Fractions (12 ml) were collected from the void volume to the maximum of haemoglobin absorbance (415 nm), pooled and designated as Fraction A. Fractions beginning at the point where A_{415} had decreased to 0.1 was assayed and those showing maximal DF_B activity (designated Fraction B) were used in experiments described herein after 5-fold concentration by filtration through a Diaflo Membrane (type YM5, Amicon Corp.).

Enzymic reduction of Fraction B and subsequent alkylation of reduced products

To avoid inactivation of the reductase in Fraction A, the experiment was carried out in two stages, as follows: (*a*) enzymic reduction of Fraction B by Fraction A and NADPH, and then (*b*) alkylation of the thiols in the reaction mixture by iodo[^{14}C]acetamide. For stage (*a*), 50 μl of NADPH (100 μM), 50 μl of cytosolic Fraction A (100 μg of protein), 100 μl of Fraction B (50 μg of protein), and 50 μl of PB-EDTA in a total volume of 250 μl were incubated at 30 °C for various times to establish when reduction had reached completion. For stage (*b*), 10 μl of iodo[^{14}C]acetamide (104 μM, 60000 c.p.m./nmol, in PB-EDTA) was added to the reaction mixture obtained in stage (*a*) and incubations were carried out at room temperature (22 °C) for various times to establish when alkylation had reached completion. Alkylation was stopped by the addition of 10 μl of cysteine (2 mM) and incubation was continued for 30 min at 22 °C. The whole reaction mixture (270 μl) was then fractionated by gel filtration on Sephadex G-50. When alkylation was carried out in the presence of 4.5 M-guanidinium chloride [15] no increase in ^{14}C incorporation was observed and thus the experimental data were obtained without the addition of this denaturant.

Separation of alkylated products on Sephadex G-50

The various ^{14}C-labelled components of Fractions A and B, as well as *S*-carboxamido[^{14}C]methylcysteine (derived from unreacted iodo[^{14}C]acetamide) were separated by chromatography of the above reaction mixture on a Sephadex G-50 column (0.6 cm × 25 cm) and elution with PB-EDTA. Fractions (250 μl) were collected at 1 min intervals directly into scintillation vials (7 ml capacity). In a control experiment, a mixture of the same concentration of cysteine (74 μM) and iodo[^{14}C]acetamide (4 μM) in a volume of 270 μl, when incubated at 22 °C for 30 min and chromatographed on the above column, gave almost 100% recovery of ^{14}C-labelled material. The ^{14}C content of the eluted fractions containing alkylated reaction products was measured by scintillation counting after mixing each with 1.25 ml of scintillation fluid consisting of 2 vol. of toluene containing PPO (0.5%) and POPOP (0.03%) plus 1 vol. of Triton X-100. The total counts associated with components in the high-M_r region (due to Fraction A), intermediate-M_r region (due to Fraction B), and low-M_r region (due to alkylated cysteine) in the elution pattern (shown in Fig. 1) were summed in each case and expressed in terms of total ^{14}C-labelled product in subsequent experiments.

Other experimental procedures

Samples were assayed for DF_B activity using Fraction A, NADPH, washed microsomes and [^{125}I]rT_3 as described previously [12]. Protein was determined by the

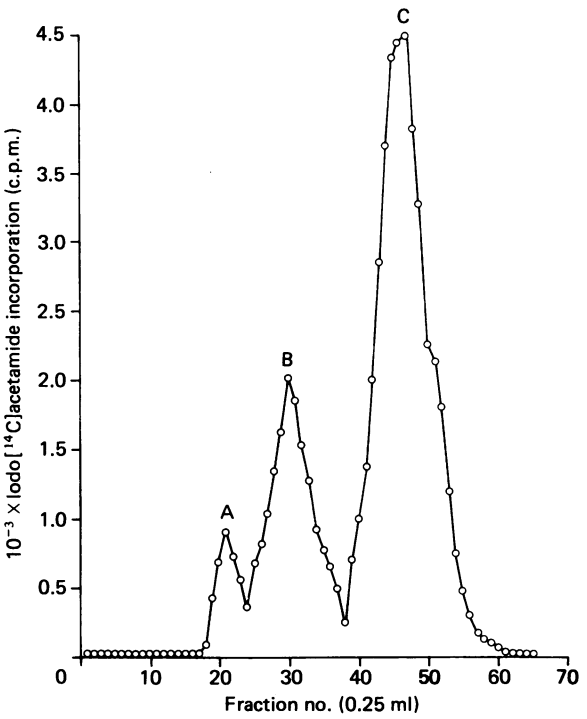


Fig. 1. Typical separation pattern obtained by chromatography on Sephadex G-50 of various ^{14}C-labelled components prepared by incorporation of iodo[^{14}C]acetamide (alkylation) into rat liver cytosolic Fractions A and B following incubation with NADPH

After the enzymic and alkylation steps (see the text) the ^{14}C (c.p.m.) of each fraction was plotted after subtracting background c.p.m. Peaks A and B, ^{14}C-labelled products derived from Fractions A and B, respectively; peak C, *S*-carboxamido[^{14}C]methylcysteine.

method of Lowry *et al.* [16] with bovine serum albumin as standard. Chemical reduction was carried out using 50 μg of Fraction B in 100 μl of PB-EDTA and 150 μl of 6.7 μM-$NaBH_4$ in 25 mM-NaOH in a total incubation volume of 250 μl. The pH was adjusted to approx. 12 and the reaction mixture was incubated at 30 °C for 2 h. The excess BH_4^- was removed by acidification with a minimal volume of HCl, followed by readjustment of pH to approx. 7.4. The whole mixture was then subjected to alkylation by iodo[^{14}C]acetamide (8 μM), the radiolabelled products were fractionated by Sephadex G-50, and the ^{14}C content of each fraction was determined as described above.

RESULTS

Chromatographic separation of alkylated components

Fig. 1 shows a typical separation pattern of the ^{14}C-labelled alkylated products obtained by reaction of Fraction B with NADPH in the presence of cytosolic Fraction A and separated by chromatography using Sephadex G-50. Three components were found, consisting of peak A (a minor component, presumably the result of alkylation by iodo[^{14}C]acetamide of some intrinsic thiol groups present in cytosolic Fraction A), peak B (the product of ^{14}C incorporation into cytosolic Fraction B), and peak C (the remaining iodo[^{14}C]acetamide, appearing as *S*-carboxamido[^{14}C]-methylcysteine). The total ^{14}C activity of component A was found to remain constant (at approximately 3000 c.p.m.) irrespective of the presence or absence of either NADPH or Fraction B in the system.

Optimization of conditions for reduction and alkylation

Preliminary experiments were performed to establish the optimum incubation times for the enzymic reduction of Fraction B in the presence of A and NADPH as well as the subsequent alkylation using iodo[^{14}C]acetamide. As shown in Fig. 2(*a*), the optimum time for enzymic reduction was 20 min at 30 °C, and for alkylation the optimum time was 90 min at 22 °C, as shown in Fig. 2(*b*). A true zero time for reduction (Fig. 2*a*) as a control could not be accomplished since the present method utilized sequential reduction and alkylation and the alkylation step required a relatively longer time to reach completion. A significant enzymic formation of thiol groups could have proceeded during the alkylation period, even in the presence of iodoacetamide. In a separate experiment (results not shown), when the reduction and alkylation were commenced at the same time at 22 °C using the combined incubation time (110 min) of the above two steps as normally used, it was found that alkylation proceeded to the extent of about 20% of that formed under standard experimental conditions (i.e. using incubation periods of 20 min and 90 min separately). Addition of an 18-fold molar ratio of cysteine to iodoacetamide was found to consume fully the amount of alkylating agent used within 30 min at 22 °C (as described under 'Materials and methods'). Thus, all iodoacetamide which remained in excess after alkylation of reduced Fraction B in the standardized method was eliminated by the addition of cysteine. Accordingly, we have assumed that the total ^{14}C-labelled products recovered in peak B following reduction, alkylation, and separation by gel-filtration chromatography was a valid measure of the extent of enzymic reduction.

Fig. 2(*a*) also indicates that by increasing the first incubation period in order to follow the progress of enzymic reduction of B a negligible effect was found on the extent of iodo[^{14}C]acetamide incorporation into Fraction A. On the other hand, an increase in the duration of alkylation (see Fig. 2*b*) slowly increased the extent of labelling of Fraction A.

Illustrated in Fig. 3 are the results obtained using the optimum reaction conditions (described above) for enzymic reduction and alkylation of Fraction B, and the data may be summarized as follows. (*a*) Fraction A incorporates an almost constant amount of ^{14}C (approx. 3000 c.p.m.) but this is abolished by heating; (b) Fraction B incorporates a larger amount of ^{14}C (approx. 15000 c.p.m.) than does Fraction A but only when both NADPH and Fraction A are also present; (*c*) heating (without subsequent centrifugation) or alkylation of Fraction A abolishes alkylation of Fraction B, but heating of Fraction B does not; and (*d*) with 200 μM-NADH, the extent of alkylation was only 8% of that obtained with 20 μM-NADPH (Fig. 3*i*).

Fig. 4 illustrates the effect of NADPH concentration on the reduction of Fraction B in terms of radiolabelled product formation. Using previously described concentrations for Fractions A and B and the established optimal time periods for the two steps, ^{14}C incorporation

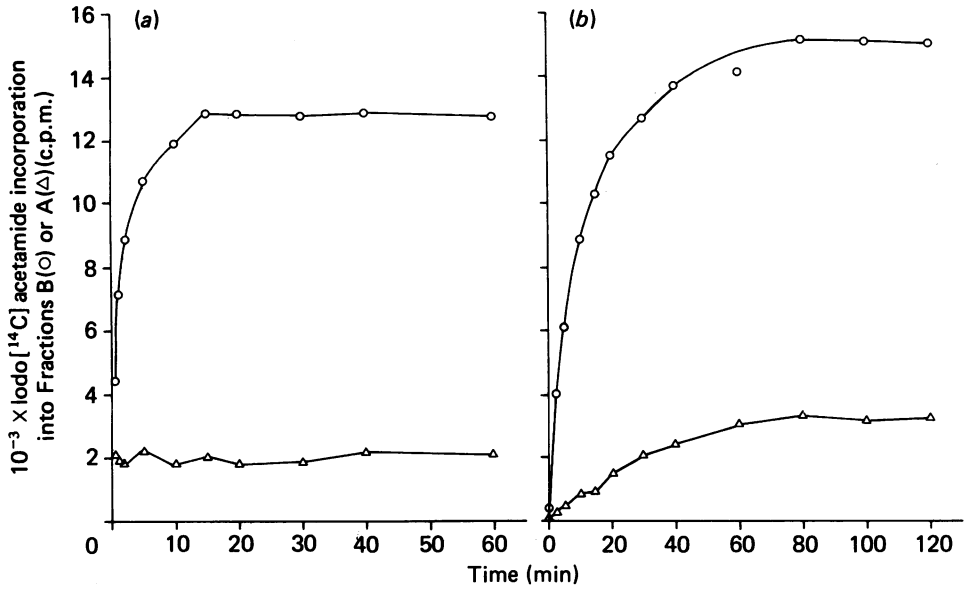
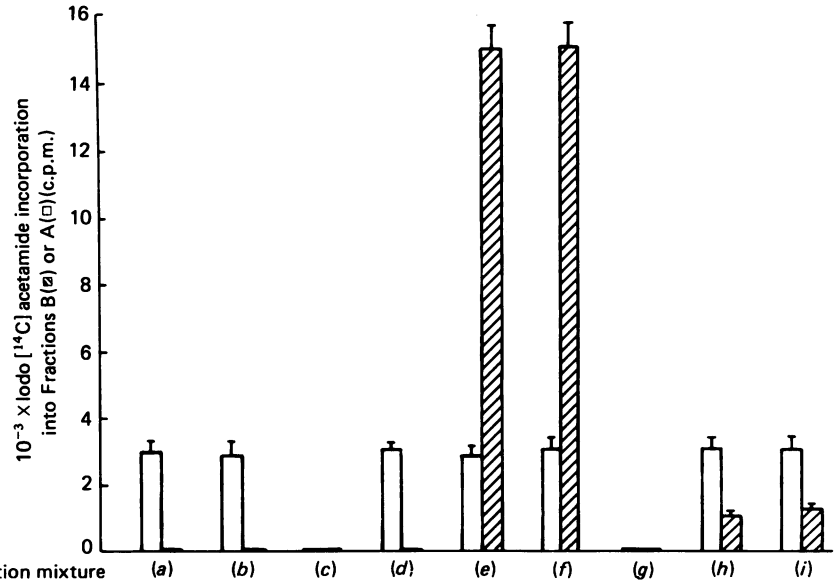


Fig. 2. Time course for NADPH-dependent enzymic reduction of Fraction B and subsequent alkylation with iodo[^{14}C]acetamide

Experimental procedures were same as described in text except in (*a*) where alkylation at 22 °C was performed for 30 min and in (*b*) enzymic reductions were carried out at 30 °C for 20 min (see the text) before each incubation period of alkylation. Each point on the curves represented the total ^{14}C (c.p.m.) under the area of peak A or peak B (see Fig. 1) obtained in each experiment. Results are the average of four determinations.



Reaction mixture	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)
Fraction A (unheated)	+	+	−	+	+	+	−	−	+
Fraction A (heated)*	−	−	−	−	−	−	+	−	−
Fraction A (alkylated)†	−	−	−	−	−	−	−	+	−
Fraction B (unheated)	−	−	+	+	+	−	+	+	+
Fraction B (heated)*	−	−	−	−	−	+	−	−	−
NADPH	−	+	+	−	+	+	+	+	−
NADH‡	−	−	−	−	−	−	−	−	+
Iodo[^{14}C]acetamide	+	+	+	+	+	+	+	+	+

Fig. 3. Enzymic reduction and alkylation under various experimental conditions

The enzymic reduction and alkylation were done using various reaction mixtures and optimum reaction conditions for reduction and alkylation (20 min at 30 °C and 90 min at 22 °C, respectively) (see Fig. 2 and text). A solid line just above the abscissa in each case represents insignificant counts above the background. Results are the average of four determinations ± S.E.M. *Fractions A and B were heated at 100 °C for 5 min before reduction and used without centrifugation. †Fraction A was preincubated with iodo[^{14}C]acetamide at 22 °C for 90 min before addition of Fraction B and NADPH. ‡200 μM-NADH was used in place of 20 μM-NADPH.

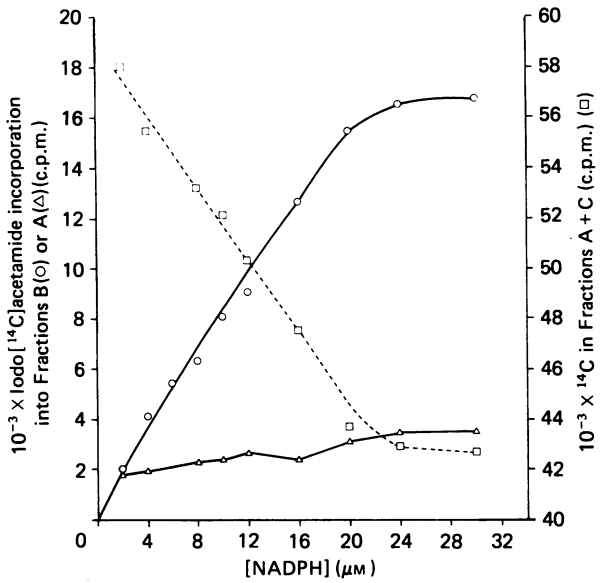


Fig. 4. Effect of increasing concentrations of NADPH on the alkylation of Fraction B following reduction

Experimental conditions were as in the text. Fraction C is *S*-carboxamido[^{14}C]methylcysteine (see peak C in Fig. 1). Results are the average of four determinations.

in peak B was found to be linear up to an NADPH concentration of almost 20 μM; a plateau was reached at 28–30 μM. In the linear range the molar ratio of NADPH to iodoacetamide incorporated can be calculated to be approx. 1:0.08. There was also a slight but progressive increase in the extent of alkylation in peak A with an increase in concentration of NADPH. It can also be seen from Fig. 4 that by plotting the sum of values (c.p.m.) of total unreacted iodo[^{14}C]acetamide (peak C) plus ^{14}C-labelled peak A (as shown in Fig. 1) against the corresponding NADPH concentrations, a reciprocal relationship with respect to alkylated reduced B could be obtained. The results, therefore, strongly suggest that the generation of thiol groups in B not only required the presence of NADPH and reductase in A, but also was directly proportional to NADPH concentration.

Effect of inhibitors and reductants other than NADPH

The addition of 0.2 mM-sodium arsenite to the enzymic reduction mixture (i.e. at the end of the first incubation and before alkylation) resulted in a dramatic (92%) reduction in the incorporation of iodo-[^{14}C]acetamide into B. On the other hand, in marked contrast with the effect of this dithiol inhibitor, the addition of iopanoate (an inhibitor of 5′-DI) had no effect on thiol generation in Fraction B (as measured by ^{14}C incorporation and compared with the control) even at a concentration of 20 μM, a 4-fold higher concentration than that used by other workers to inhibit 5′-DI [16]. Incorporation of ^{14}C into Fraction A was also not affected by prior incubation with iopanoate (results not shown). A similar lack of inhibition was observed with 1 mM-PTU regardless of whether it was present during enzymic reduction, alkylation or even during preincubation with Fraction A. The level of ^{14}C-labelled products observed in peak B under all experimental conditions remained constant at about 15000 c.p.m.

Studies on the reduction of Fraction B using a monothiol (GSH), a dithiol (DTT) and $NaBH_4$ (each at 4 μM) as alternate reductants in place of NADPH and Fraction A prior to alkylation are illustrated in Fig. 5. DTT and $NaBH_4$ were found to be capable of reducing Fraction B to a similar degree whereas GSH, a weak activator of 5′-DI as previously demonstrated in our laboratory [12,13], was completely ineffective at 4 μM. However, in terms of ^{14}C incorporation the reducing potencies of DTT and $NaBH_4$ were 2.5–2.8 fold greater than that obtainable using similar concentrations of NADPH (4 μM) and Fraction A (Fig. 5).

Loss of 5′-DI activation by Fraction B following its partial reduction and alkylation

Fig. 6 shows the results of an experiment designed to study the correlation between the potentiating effect on 5′-DI by the various concentrations of Fraction B which remained unreduced (and therefore not capable of being alkylated) after various degrees of partial reduction and alkylation and the indicated concentration of NADPH used for partial reduction. Unlabelled iodoacetamide at 4 μM was employed in these studies and the various amounts of residual (unalkylated) Fraction B were determined by their capacity to stimulate 5′-DI in the usual assay [2,12] (Fig. 6). A progressive decrease in 5′-DI activation capacity by Fraction B was observed as the NADPH pretreatment concentration was increased. Comparison of the data in Fig. 6 with those in Fig. 4 shows a reciprocal relationship, indicating that the potentiating effect of Fraction B on 5′-DI corresponds to the amount of residual active disulphide groups present

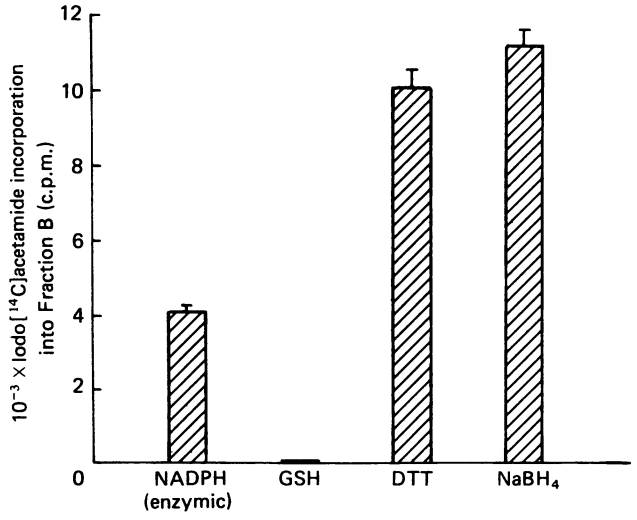


Fig. 5. Effect of GSH, DTT and $NaBH_4$ as alternative reductants for Fraction B

Fraction B was reduced either enzymically using NADPH in the presence of Fraction A (reductase) or by alternative means using GSH, DTT or $NaBH_4$ (4 μM in each case) in place of NADPH and A as indicated. Iodo[^{14}C]acetamide was used in each case for alkylation at 8 μM (sp. radioactivity 60000 c.p.m./nmol). Reduction with GSH and DTT was carried out at 30 °C for 30 min. Chemical reduction using $NaBH_4$ and other reaction conditions including the protein concentrations of A and B are described in the text. Results are the average of four determinations ± S.E.M..

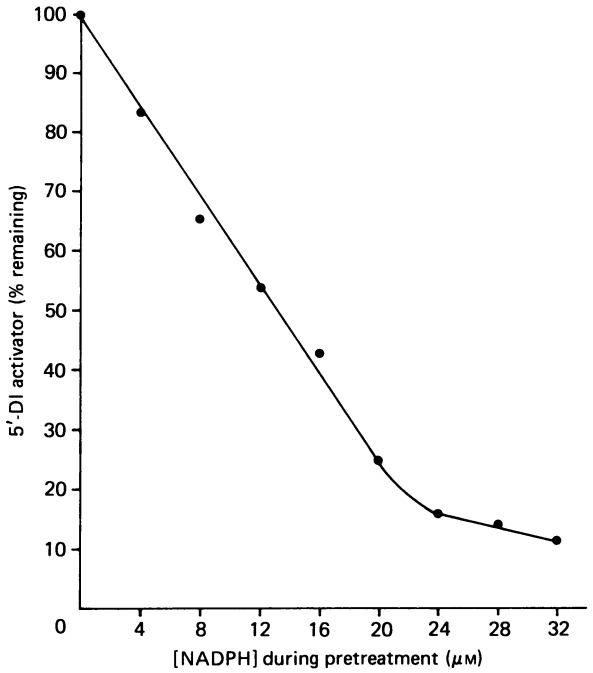


Fig. 6. Determination of residual 5'-DI activator activity after partial reduction of Fraction B and inactivation by alkylation

Nine separate enzymic reductions of Fraction B (50 μg each) were carried out in the presence of fraction A (100 μg each) using increasing but limiting concentrations of NADPH (as indicated on the abscissa) in a total incubation volume of 250 μl in PB-EDTA as described in the text. The individual reduced mixtures were alkylated by unlabelled iodoacetamide (4 μM) followed by addition of an amount of cysteine equivalent to the iodoacetamide and incubation for 1 h at 22 °C. To each reaction mixture an additional amount of Fraction A (50 μg), NADPH (80 μM), microsomal protein (14 μg of 11.2 mg/ml) and [^{125}I]rT$_3$ (0.21 nM, 100000 c.p.m.) were added in a final volume of 500 μl and the determination of 5'-DI activator activity was carried out as previously described [12]. The extent of ^{125}I released/15 min was determined after subtracting an appropriate blank value. The residual activator activity of partially inactivated Fraction B in each case was expressed as a percentage of control (prepared with same amounts of Fraction B but without prior reduction and inactivation by iodoacetamide. 100% activator activity = 24 fmol of [^{125}I]rT$_3$ deiodinated/15 min. Results are the average of four determinations.

which had remained in an unreduced form and was therefore not inhibited by iodoacetamide. To neutralize unreacted iodoacetamide which would interfere with a microsomal 5'-DI assay [17], an amount of cysteine equivalent to the iodoacetamide was added before performing the 5'-DI assay. A mixture of 4 μM each of cysteine and iodoacetamide incubated at 22 °C for 1 h was shown in control experiments to have no effect on 5'-DI activation (result not shown).

DISCUSSION

In a previous report from this laboratory [12] the activation of 5'-DI by a rat liver cytosolic component (DF_B) of M_r approx. 13000 was found to be dependent upon the simultaneous presence of a second cytosolic component (DF_A) of $M_r > 60000$ and NADPH [12]. DF_B could also enhance 5'-DI activation when DF_A and NADPH were replaced by various thiols, with the dithiols DTT and dihydrolipoamide being more potent than the monothiols 2-mercaptoethanol and GSH [13]. In explanation of these findings it was proposed that DF_B contains a disulphide which requires reduction in order to become an activator of 5'-DI [13]. The present study sought to determine whether this mechanism is correct by measuring the generation, under various conditions, of thiol residues through their incorporation of a radiolabelled alkylating agent.

Under all conditions tested incorporation of ^{14}C was influenced by the same factors and in the same manner as the activation of 5'-DI by Fraction B (containing DF_B) reported earlier [12]. Thus labelling of Fraction B was found to have an absolute requirement for both Fraction A and NADPH (Fig. 3) and to be directly proportional to the concentration of NADPH added (Fig. 4). Furthermore, conversion of increasing amounts of Fraction B components to the reduced form, which could thereby be inactivated by alkylation, produced a proportionate loss of residual 5'-DI activator activity upon incubation with additional Fraction A and NADPH, as well as microsomes (Fig. 6.) Heat treatment of Fractions A and B (without subsequent centrifugation) produced quite different effects upon the observed ^{14}C incorporation into Fraction B components and showed that Fraction A was heat-labile whereas Fraction B was stable (Fig. 3); the same differential susceptibility of these fractions to thermal denaturation was demonstrated in previous studies of 5'-DI activation reported from our laboratory [12].

Inhibition by arsenite of ^{14}C incorporation into enzymically reduced Fraction B components not only adds further support to the proposal that thiol groups are generated during the interaction between Fraction A, Fraction B and NADPH, but also indicates that the active form of DF_B is a dithiol, having two groups in sufficiently close proximity to one another to allow formation of a stable inactive cyclic complex with arsenite [18], thus preventing alkylation. However, by comparison, PTU, which is a potent inhibitor of 5'-DI, competing with thiol activators such as DTT [19,20], had no effect on the ^{14}C incorporation into Fraction B following enzymic reduction (Fig. 5); a similar lack of effect of iopanoate (Fig. 5), another inhibitor of 5'-DI which acts by a mechanism different from PTU [21,22], was observed. Thus, PTU and iopanoate do not function as inhibitors of 5'-DI by interfering with reduction of the cofactor DF_B.

Although a linear relationship was observed between the extent of reduction of Fraction B and NADPH concentration (Fig. 4), a significant discrepancy is evident in the molar ratio between iodo[^{14}C]acetamide incorporated in the reduced product and the NADPH used. In practice, we always observed greater NADPH utilization than expected. Theoretically, 1 mol of NADPH would lead to the production of two equivalents of alkylated thiol, assuming that both reduction and alkylation are quantitative, whereas in the present study a ratio of 1:0.08 was obtained. Apparently alkylation had proceeded to completion at pH 7.4, since no further increase was obtained by incubation longer than 80 min (Fig. 2*b*) or by carrying out the alkylation in the

presence of 4.5 M-guanidinium chloride (results not shown), which was found by others [15] to be required for the alkylation of both cysteine residues of reduced thioredoxin. The high NADPH/^{14}C ratio could have resulted from the presence in Fraction A of a previously reported NADPH oxidase [14] as well as haemoglobin, which is known to cause oxidative breakdown of NADPH [24,25]. These factors may have caused the pronounced deviations from expected stoichiometry.

The reductase DF_A (a constituent of chromatographic Fraction A) is apparently sensitive to iodoacetamide (Fig. 3, *h* versus *e*) and therefore may contain one or more essential thiol residues. Fig. 3 also indicates that the reduction of DF_B by DF_A and dinucleotides is highly specific for NADPH relative to NADH. The greatly increased incorporation of ^{14}C into Fraction B following reduction by DTT or $NaBH_4$ rather than NADPH (Fig. 5) indicates that approx. 40% of the available disulphide bonds are susceptible to specific enzymic reduction by NADPH.

In contrast with our recent observation of an NADPH-dependent and GSH-independent 5′-DI cytosolic cofactor (M_r approx. 13000), Goswami & Rosenberg [26] isolated a soluble basic protein, SPF, of M_r 11000 which was found to stimulate 5′-DI only in the presence of 5 mM-GSH and was virtually insensitive to PTU. However, the exact physiological relevance of that system, in which the concentrations of iodothyronine substrate employed were unphysiological, has not yet been definitely established. Evidence suggesting a close similarity between SPF, glutaredoxin and thiol transferase has been presented [26,27], but since these cytosol components are active only in the presence of GSH (which in our studies has been chromatographically removed [12] from Fraction B), the 5′-DI activation system presently under study does not involve these components.

The present report provides direct confirmation of previous observations from our laboratory [12–14] and demonstrates the existence of a cytosolic disulphide (DF_BS_2) in Fraction B which can be reduced to a dithiol [$DF_B(SH)_2$] enzymically by physiological concentrations of NADPH (20–60 μM) in combination with a cytosolic Fraction A. The data obtained previously [12,13] as well as in the present investigation on the properties of DF_B, including an M_r of approx. 13000, a pI < 6 by chromatofocusing (A. K. Das, B. C. W. Hummel & P. G. Walfish, unpublished work), activation by an enzyme of high M_r operating in conjunction with NADPH during which a dithiol is generated, heat stability, and finally potentiation of the effect of DTT and other thiols [13] have, in fact, not differentiated DF_B from thioredoxin [28]. The possibility must therefore be considered that these substances are very similar or possibly identical. Since Fraction A contains an enzyme similar to thioredoxin reductase, because of its ability to reduce Nbs_2 [23] (A. K. Das, B. C. W. Hummel & P. G. Walfish, unpublished work), ^{14}C appearing in peak B (Fig. 1) may well represent alkylated thioredoxin as well as alkylated DF_B, a substance of similar properties. Thus, thioredoxin may have contributed significantly to the activation of 5′-DI by cytosolic Fraction B in which nanomolar rT_3 and micromolar NADPH concentrations were employed, as reported previously [12,13].

It was recently reported that at micromolar concentrations of iodothyronine substrate the thioredoxin system failed to stimulate 5′-deiodination [26]. Nevertheless, recent investigations in our laboratory [29] on the effect of starvation on the changes in 5′-DI activation (measured using nanomolar rT_3 concentrations) by hepatic microsomal and cytosolic components have shown a direct correlation between the changes in 5′-DI-supportive activity of whole cytosol and that which could be obtained from cytosolic Fraction B. Changes in iodo[^{14}C]acetamide-alkylated products obtainable from Fraction B during starvation also correlated with its supportive activity (K. Iwase, B. C. W. Hummel & P. G. Walfish, unpublished work). Such observations attest to the physiological relevance of our previously proposed thioredoxin-like cofactor [12,13] which is operative in a deiodination system when a low (nanomolar) iodothyronine substrate concentration is utilized.

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